Inhibition of Herpes Simplex Virus-1 Entry into Human Cells by Nonsaccharide Glycosaminoglycan Mimetics

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Supporting Information

ABSTRACT: Although heparan sulfate (HS) has been implicated in facilitating entry of enveloped viruses including herpes simplex virus (HSV), small molecules that effectively compete with this abundant, cell surface macromolecule remain unknown. We reasoned that entry of HSV-1 involving its glycoprotein D (gD) binding to HS could be competitively targeted through small, synthetic, nonsaccharide glycosaminoglycan mimetics (NSGMs). Screening a library of NSGMs identified a small, distinct group that bound gD with affinities of 8–120 nM. Studies on HSV-1 entry into HeLa, HFF-1, and VK2/E6E7 cells identified inhibitors with potencies in the range of 0.4–1.0 μ M. These synthetic NSGMs are likely to offer promising chemical biology probes and/or antiviral drug discovery opportunities.

KEYWORDS: Chemical probes, glycoprotein D, glycosaminoglycans, heparan sulfate mimetics, herpes simplex virus

Herpes simplex virus type-1 (HSV-1), a member of the alphaherpesvirus family, is a ubiquitous human pathogen causing a spectrum of diseases ranging from cold sores on mucosal layers of the skin of the mouth and face to much more severe, life-threatening illnesses, such as keratitis, encephalitis, and meningitis.¹ Among the two serotypes, HSV-1 is primarily associated with the orolabial ulceration; however, epidemiological studies have shown an increase in genital and neonatal herpes caused by HSV-1.^{2,3} No vaccine is available to protect against these infections. The virus also continues to develop resistance to current therapies, such as acyclovir, which highlight the need to find novel agents and pathways to fight HSV infection.⁴

HSV infection typically begins with viral glycoproteins B (gB) or C (gC) binding to heparan sulfate proteoglycans (HSPGs) that line host cell membranes.^{5–7} In addition, three viral glycoproteins have been implicated in the viral entry process including gD, gH, and gL. Of these, the participation of gD is thought to be very important,^{5–11} especially because a rare 3-O-sulfated HSPG has been implicated in its interaction by a number of groups.^{7,8,12,13} In addition to HSPG on cell surfaces, gD interacts with two other cellular receptors, including herpes virus entry mediator (HVEM) and nectin-1,^{9,11,14,15} that trigger a cascade of events eventually leading to viral entry.

Although all three receptors (HSPG, HVEM, and nectin-1) are putative targets for designing anti-HSV agents, the gD–HSPG interaction appears to be easier to target because numerous mimetics of HS have been studied as antiherpesvirus entry agents.^{5,10} Majority of these polymeric mimetics are derived from sea algae¹⁶ including the carrageenans, galactofucan sulfate, and fucoidan. Some synthetic mimetics of HS have also been investigated including nonanticoagulated heparin,¹⁷ pentosan polysulfate,¹⁷ dextran sulfate,^{17,18} and low molecular weight lignins.^{19,20} Generally, these polysaccharides display reasonable to good inhibition profiles. However, these polymers are unlikely to succeed as clinical anti-HSV candidates because of their massive heterogeneity and difficulty of synthesis. Also, identifying the preferred viral target of these sulfated polymers is very difficult.

We reasoned that screening a class of small molecules, called nonsaccharide glycosaminoglycan (GAG) mimetics (NSGMs), against gD could help identify new anti-HSV agents. NSGMs are much smaller than polymeric heparan sulfate mimetics.²¹ NSGMs are easy to synthesize and highly water-soluble. More importantly, NSGMs are functional mimetics of polymeric

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GAGs, a function that arises from their binding to GAG-binding sites on proteins. A number of NSGMs have been discovered/ designed so far. These include the sulfated flavonoids and xanthenes as anticoagulant and antiplatelet agents,^{22,23} sulfated benzofurans as inhibitors of thrombin,²⁴ and sulfated benzylated glycosides as inhibitors of factor XIa.^{25,26}

This work shows for the first time that as a novel class of GAG mimetics, NSGMs, present excellent alternatives for inhibiting HSV. The work identifies distinct NSGMs from a small library that bind gD with high affinity and inhibit cellular entry of HSV-1 with IC₅₀ in the range of 430 nM to 1.0 μ M, which is more than 10-fold lower than that reported for 3-O-sulfate-containing heparin/heparan sulfate-derived octasaccharides.^{27,28}

Several lines of evidence suggest that gD could be effectively targeted by sulfated agents. First, the crystal structure of gD shows a large constellation of Arg and Lys residues that can bind to multiple sulfate groups. In fact, two sulfate anions are present in crystals.⁹ Second, mutagenesis reveals a pocket proximal to the N-terminus as important for HS binding.²⁹ Third, a 3-O-sulfated octasaccharide with an affinity of 18 μ M has been identified as the minimal gD binding sequence.²⁷ Two additional octasaccharides were also found to inhibit the virus.²⁸

We hypothesized that gD may preferably engage much smaller, sulfated molecules than polymeric sulfated biomolecules. To test this, we studied a library of 13 sulfated saccharides and NSGMs (Figure 1). The saccharides included sucrose octasulfate (SOS, 1), fondaparinux (2), unfractionated heparin (UFH, 3) and chondroitin sulfate A (CSA, 4). The NSGM group included nine molecules based on different scaffolds including flavonoid-based agents 5 and 6,³⁰ benzofuran-based agents 7 and 8,³¹ and glycoside-based agents 9–13.^{25,26,32}

Of these, agents 1, 2, 5–8, and 10–13 are homogeneous, per-sulfated molecules (>95% purity) as deduced using reversed-phase ion-pairing UPLC-ESI-MS. In contrast, 3 and 4 are natural, commercially available biopolymers with considerable structural variation, and 9 is a synthetic molecule described in multiple articles as a promising anticoagulant devoid of enhanced bleeding risk.^{25,26,33} Detailed studies have shown that 9 is composed of six species (hepta- to dodeca-sulfated), of which the deca-sulfated species is the most common.^{26,27} The average molecular weight (M_R) of 9 has been calculated to be 2178,²⁵ whereas the M_R of 3 and 4 are known to be ~15,000 and ~25,000, respectively. Thus, NSGMs 5–13 are much smaller in comparison to biopolymers 3 and 4, perhaps by ~600%.

With regard to the number of sulfate groups, NSGMs carry two to 15 sulfates on their small scaffolds, whereas biopolymers 3 and 4 carry some 80 and 60 sulfates, respectively, per chain. In combination, NSGMs 5–13 and saccharides 1–4 afforded good diversity of core scaffolds, three-dimensional orientation of sulfate groups, and sulfation levels, which in principle should enhance the probability of tight binding ligands for gD.

Fluorescence-based methods have been typically used for measuring affinities of sulfated agents for their targets. For example, NSGM 9 induced a decrease in the intrinsic fluorescence of factor XIa,^{25,26} whereas sulfated quinazolinones induced an increase in fluorescence of dansylated factor XIa.³⁴ Using intrinsic fluorescence titrations in pH 7.4 buffer at 37 °C (λ_{EM} = 333 nm), we found that each library member bound to gD (Figure 2 and Supplementary Figure S1). Standard nonlinear binding equation gave K_D s in the range of 8 to 2230 nM for 1–13 (Table 1).

Among the saccharides, 1 and 2 showed gD affinities of 0.15 and 0.5 μ M, whereas heterogeneous biopolymers 3 and 4

Figure 1. Structures of ligands 1–13 screened for binding to glycoprotein D (gD). The library included saccharide ligands 1–4, flavonoid-based NSGMs 5 and 6, benzofuran-based NSGMs 7 and 8, and glycoside/pseudoglycoside-based NSGMs 9–13. The agents differ in type of core scaffold, number of sulfate groups, and overall three-dimensional structure.

displayed affinities of 2.23 and 1.48 μ M. Normally, statistical advantage induces longer chains to bind better to targets than smaller chains. This conveys the idea that GAG-binding domain(s) on gD are well-defined and do not prefer larger species.

The NSGMs displayed interesting characteristics. Three different core scaffolds present in the NSGMs, including the flavonoids (5 and 6), benzofurans (7 and 8), and glycosides/pseudoglycosides (9–13), displayed an affinity range spanning 8 nM to 2.22 μ M. It is important to recall NSGMs 5–8 and 11–13 are homogeneous entities, but 9 is variably sulfated. Yet, its M_R (= 2178) is close to the molecular weight of its most common component (deca-sulfated; MW = 1961). This implies that the observed affinity range (8 to 2220 nM) represents a large change of ~220-fold for molecules of the size of NSGMs, thus suggesting good structure-dependent activity. Another point of interest is

Figure 2. Spectrofluorometric titrations of gD binding to saccharides 1–4 (A) and NSGMs 9–13 (B) at pH 7.4 and 37 °C monitored by intrinsic fluorescence (see titrations of 5–8 in [Supplementary Figure S1](#)). Solid lines represent nonlinear regression to obtain K_D .

Table 1. Affinity (K_D) and Maximal Fluorescence Change (ΔF_{MAX}) of Sulfated Saccharides and NSGMs Binding to gD^a

NSGM	K_D (μM)	ΔF_{MAX} (%)
1	0.15 ± 0.07^b	-52 ± 10
2	0.50 ± 0.06	-85 ± 8
3	2.23 ± 0.26	-43 ± 4
4	1.48 ± 0.74	120 ± 25
5	0.022 ± 0.004	-82 ± 6
6	0.105 ± 0.008	-118 ± 6
7	2.22 ± 0.74	-112 ± 36
8	0.78 ± 0.19	-157 ± 32
9	0.010 ± 0.005	158 ± 22
10	0.112 ± 0.024	328 ± 44
11	0.026 ± 0.009	87 ± 12
12	0.033 ± 0.008	115 ± 22
13	0.008 ± 0.003	53 ± 8

^aMeasured using the intrinsic tryptophan fluorescence change in pH 7.4 buffer at 37 °C. See Methods in the [Supporting Information](#) for details. ^bErrors represent standard error calculated using global fit of the data.

the opposing change in gD fluorescence induced by 4 and 9–13 in contrast to the remaining NSGMs ([Table 1](#)). Although unclear at present, it is likely that the conformational change induced in gD by the two groups of NSGMs is different, which may or may not have functional consequences.

Flavonoid-based NSGMs 5 and 6 carry eight sulfate groups and show affinities of 22 and 105 nM, which are much higher than those of benzofuran-based NSGMs 7 and 8 that carry two sulfate groups each ([Table 1](#)). The glycoside and pseudoglycoside

NSGMs 9–13 are fundamentally different from nearly all agents described so far. One, these contain multiple chiral centers in comparison to the other NSGMs, which carry none. Second, the glycoside or pseudoglycoside scaffold affords a more globular distribution of sulfate groups in comparison to other NSGM scaffolds that present a more linear organization. Third, the number of sulfate groups on these scaffolds (6 to 15) is also higher than other scaffolds (2 and 8). The gD affinities of these NSGMs ranged from 8 to 112 nM ([Table 1](#)), of which 9 and 13 were most potent at 10 and 8 nM, respectively.

Overall, several points are worth noting. (1) Generally, sulfated NSGMs bind better than biopolymers 3 and 4 by orders of magnitude, although NSGMs 7 and 8 are particularly disfavored by gD. (2) The glycoside/pseudoglycoside group (NSGMs 9–13) displays higher affinities than the other two groups. (3) In fact, 13 and 9 display gD binding affinities that reflect therapeutic promise. Of the two, 13 is homogeneous, whereas 9 is a mixture showing affinity equivalent to 13. These agents represent the most potent small, sulfated NSGMs known to bind to their targets.^{23–26,30–32,35}

To understand gD recognition by NSGMs, we first analyzed its x-crystal structure. gD carries ₁₈₂EHRAKG₁₈₇ sequence that matches the Cardin–Weintraub consensus GAG-binding XBBXB sequence (X = hydrophobic, B = basic residues), which presumably forms the site of GAG/NSGM binding. To test this, we calculated the electrostatic surface potential of gD, which identified two sites with highly electropositive surface. Subsite 1 arises from contributions of K₁, R₃₅, R₁₃₀, R₁₃₄, and R₂₂₉, while subsite 2 is brought about by R₆₄, R₆₇, H₁₈₃, R₁₈₄, K₁₈₆, R₁₉₀, K₂₃₇, and R₂₄₅ ([Figure 3A](#)). Interestingly, a short “mound” between the two subdomains did not bear much electropositive character. Considering that nothing is known about which of these residues are engaged by GAGs, we defined all residues within 32 Å of R₁₃₀ (site 1) and K₂₄₅ (site 2) as the site of binding for our docking studies. We utilized GOLD-based protocol to study GAG binding to proteins.^{30,36–39} This protocol utilizes a genetic search algorithm that attempts to identify the best possible interaction pose(s) of the ligand through an exhaustive series of unbiased mutagenesis of interaction geometries encompassing the entire binding region. We developed this protocol in 2006⁴⁰ to elucidate how molecules as complex as GAGs bind to their targets.³⁶ We have found this protocol to be also useful for sulfated NSGMs.^{35–39}

NSGMs 5–8 and 10–13 were modeled in their homogeneous form, whereas 9 was simulated in its most prevalent decasulfated form (X = SO₃[−], Y = OH, [Figure 1](#)) as well as the per-sulfated form (X = SO₃[−], Y = OSO₃[−], [Figure 1](#)). [Figure 3B](#) shows compilation of binding poses for NSGMs 9–13, which suggests reasonable overall consistency of interactions for the most potent molecules, especially for subsite 1. NSGMs from the benzofuran and flavonoid groups displayed GOLDScores in the range of 76–120, whereas those from the glycoside/pseudoglycoside group were higher (107–156) supporting tighter binding. NSGM 9 in the deca-sulfated form displayed GOLDScores of 137 for subsite 1, whereas 13 showed a score of 131 ([Table S1](#)). The similarity of these interaction scores arises from the similarity of their predicted binding poses ([Figure S2](#)).

To infer the overall predictability of modeling, the scores were plotted against binding affinities ([Figure 3C,D](#)). The plots showed reasonable correlation coefficients (R^2) of 0.79 and 0.58, respectively. This implies that subsite 1 is more preferred over subsite 2; but we should be cautious in oversimplification. It is possible that both sites may be engaged simultaneously. In fact,

Figure 3. (A) Electrostatic potential surface map of gD calculated using APBS tool in PyMol. Blue and red represent electropositive and electronegative surfaces, respectively. Two potential GAG binding regions, subsites 1 and 2, are predicted. (B) Overlay of docked poses of 9–13 obtained from GOLD-based docking onto subsites 1 and 2 of gD (ribbon). (C,D) Correlations between GOLDScore and binding affinity for subsites 1 and 2, respectively. Colored-coded symbols include saccharides 1 and 2 in gray; flavonoid-based NSGMs 5 and 6 in blue; benzofuran-based NSGMs 7 and 8 in yellow; and glycoside-based NSGMs 9–13 in orange.

stoichiometry of 9–gD complex, measured using spectrofluorimetry, was found to be 1.7:1.0 (Figure S3), which suggests complete engagement of both sites simultaneously.

More in-depth analysis clarifies the atomistic reasons for the high affinity binding of NSGMs of 9 and 13. The agents utilize hydrogen bonding as well as hydrophobic (π – π ; π –alkyl) interactions with several basic and polar residues (Figure S2). More importantly, specific sulfate groups on NSGMs 9 and 13 interact with R_{36} , R_{82} , R_{130} , N_{148} , and R_{229} (Figure S2). These interactions are much weaker or nonexistent for NSGMs 11 and 12. This implies that analogs of these NSGMs, especially 13, that retain these key sulfate groups are likely to yield more potent gD ligands. One example of such an analog, designed on the basis of the observed key interactions, is shown in Figure S4. The analog presents minimal number and position of sulfate groups necessary for tight binding to gD.

Because NSGM 9 was the easiest to synthesize on larger scale and equivalent in gD affinity to NSGM 13, we studied its anti-HSV activity in HSV-1 entry assay. We have earlier used a reporter-based viral entry assay to assess the antiviral potential of different polymers.^{19,20,41} In this assay, HeLa, HFF-1, and VK2/E6E7 cells were exposed to a reporter HSV-1 containing the lacZ gene in the presence of NSGMs. Theoretically, binding of 9 to gD would competitively prevent the virus from binding to HSPG and other receptors on the target cells, thereby reducing viral entry. This in turn would reduce the β -galactosidase activity arising from the internalized viral particle. Figure 4A shows the reduction in β -galactosidase activity as a function of 9. Analysis of the profile using the standard dose–response equation led to an IC_{50} of 0.43 μ M (HeLa), 0.49 μ M (HFF-1), and 1.0 μ M (VK2/E6E7). These activities compared favorably with the activities of full-length heparin (see Figure S5).

To determine the impact of 9 on cellular toxicity, an LDH cytotoxicity kit was utilized to measure LDH release at three different time points in various cell lines. Figure 4B shows the results for cells exposed to 9 for 24 h. The results revealed that 9 is completely nontoxic to human cells below 100 μ M and especially at the concentrations needed to inhibit HSV-1 entry into cells. Most importantly, the level of inhibition of HSV-1 cellular entry is >12-fold better than that observed earlier with heparin octasaccharides.^{27,28} We also studied the influence of 9 in HSV-1 plaque formation. In this assay, Vero cells were pretreated with 9, exposed to HSV-1 KOS, and then allowed to grow in the presence of 9 or acyclovir or mock treatment. The plaques formed at the

Figure 4. (A) Colorimetric assay for detection of HSV-1 entry into HeLa, HFF-1, and VK2/E6E7 cells in the presence of 9. Cells were pretreated with 9 for 1 h at 37 °C and 5% CO_2 , washed, and challenged with HSV-1 KOS (gL86) for 6 h. Viral entry corresponds to expression of β -galactosidase activity. (B) Cellular toxicity of 9 measured using lactate dehydrogenase (LDH) activity released upon damage of plasma membrane. (C) Reduction of HSV-1 plaque formation in the presence of 9 and/or acyclovir (Ac. = acyclovir). See text for details.

end of 72 h period were counted (Figures S6). Figure 4C shows the inhibition of plaque formation in the presence of 10 and 100 μ M 9. Both concentrations reduced plaque formation suggesting high antiviral effect. The activity was comparable to that of acyclovir, the anti-HSV drug currently in clinical use, and could be augmented further with 9.

The results presented above show for the first time that sulfated small molecules can potentially inhibit HSV-1. NSGMs are much smaller than HSPGs present on the cell surface. NSGMs are also synthetic agents in contrast to all natural sulfated biopolymers. The anti-HSV potency observed in this study (0.43 to 1.0 μ M) is highly indicative of promising drug-like characteristics.

To develop sub-nM NSGMs, advanced approach(es) will have to be developed. The gD affinities measured for the glycoside-based NSGMs were $\sim$ 10–100 nM. This is in the range of the affinity of gD for nectin-1 (17 nM).¹⁴ This raises the possibility that NSGMs may competitively reduce gD–nectin-1 interaction.

One line of advanced drug discovery based on this observation would be to design agents that bear nectin-1 and/or HVEM recognizing elements. Such a dual “pharmacophore” NSGM–HVEM/nectin-1 analog(s) should display sub-nM potency. Alternatively, an NSGM and a nectin-1 binding agent could be simultaneously utilized to effect synergistic action.

In either case, developing higher potency inhibitors would be important because it is known that a single copy of viral gD can ensure target cell infectivity.⁴² This is further aided by extremely strong interactions of HSV with the host plasma membrane.⁴³ This imparts even more importance to multisite antagonistic action of gD inhibitors. NSGMs may lead to such agents by targeting multiple entry-essential glycoproteins.

Yet, achieving this is not expected to be easy. The crystal structure of NSGM–gD complex is not available and expected to be challenging. Thus, designing a dual “pharmacophore” NSGM–HVEM/nectin-1 analog is likely to be challenging but highly rewarding.

Overall, we present sulfated NSGMs as major opportunities for development of antivirals. Our work shows that (i) NSGMs as a group represent an excellent alternative to natural GAGs or polymeric GAG mimetics for targeting gD; (ii) several structurally distinct NSGMs may be useful as chemical probes in biological studies with gD; and (iii) specific NSGMs (**9** and **13**) were found to be potent inhibitors of HSV-1 entry into three different human HSV infection-related cell lines.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmmedchemlett.7b00364.

Figures S1–S6 and Tables S1 and S2 (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CSA, chondroitin sulfate; GAG, glycosaminoglycan; gD, glycoprotein D; HFF, human foreskin fibroblasts; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; HSV, herpes simplex virus; HVEM, herpes virus entry mediator; LDH, lactate dehydrogenase; NSGM, nonsaccharide glycosaminoglycan mimetic; PDB, Protein Databank; SOS, sucrose octasulfate; UFH, unfractionated heparin; VK2/E6E7, human vaginal epithelial cells

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